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(54) Title: METHOD FOR ENZYMATIC DETERMINATION OF ALDOSES

(57) Abstract

The present invention relates to a method for quantitative determination of aldoses, in particular xylose. According to the method, the aldose is enzymatically oxidized to the corresponding aldonolactone with the aid of an aldose dehydrogenase in the presence of an electron acceptor. The amount of the reduced electron acceptor is determined as a measure of the amount aldose. According to the invention, a PQQ-linked aldose dehydrogenase enzyme is used for oxidizing the aldose. The enzyme is preferably obtained from strains of the genus *Gluconobacter*. The invention also concerns a method for determining the monomeric aldose units of oligomeric sugars. By immobilizing the aldose dehydrogenase enzyme on an electrode, a aldose biosensor is provided which can be used for electrochemical methods for determination of aldoses.

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METHOD FOR ENZYMATIC DETERMINATION OF ALDOSES

FIELD OF THE INVENTION

The present invention concerns a method for quantitative determination of aldoses. According to the method, the aldose which is to be determined is enzymatically oxidized to the corresponding aldonolactone with the aid of an aldose dehydrogenase in the presence of an electron acceptor. The amount of the reduced electron acceptor is determined as a measure of the amount of aldose.

The method according to the invention can also be used for assaying oligosaccharides.

The invention also concerns an electrode for use in electrochemical methods for the determination of aldoses.

BACKGROUND OF THE INVENTION

The quantitative and specific determination of aldoses, especially xylose and glucose, is essential in biotechnology, in particular in biomass research and in food technology. Thus, in the case of xylose, which is a rather abundant aldose sugar in nature, assays are routinely carried out in many biotechnical applications, such as in xylose fermentation studies, biobleaching research and in hydrolysis studies of hemicelluloses.

At present there are several known methods for assaying aldoses. The oldest one comprises colorimetric quantification of reducing sugars by using, e.g., dinitrosalicylic acid (DNS) (Miller 1959) or arsenomolybdate (Nelson 1944, Somogyi 1952). Both of these methods are, however, very unspecific. Aldoses can be specifically measured by HPLC, but the HPLC apparatus is relatively expensive.

In order to overcome the drawbacks of the prior art, methods employing enzymes have been proposed. Enzymatic assay methods have, in general, many benefits; the apparatus costs are low and the methods are specific and fast.

Of the aldoses, glucose can be enzymatically measured by, e.g., glucose oxidase (Bergmeyer

1974), and methods for determining other monosaccharides, such as galactose, have also been developed (Bergmeyer 1974).

Although some xylose assay methods involving the use of enzymes have been published in the art, no methods have been commercialized so far.

WIssler and Logemann (1984) suggest a direct enzymatic method for xylose determination employing an NADP-linked xylose dehydrogenase isolated from pig lens. This NADP-linked xylose dehydrogenase is reported to be specific to xylose, but the reaction is an equilibrium reaction and the removal of the end-product (xylonolactone) is essential. Wissler and Logemann (1984) claim that the irreversibility of the reaction is obtained by the spontaneous hydrolysis of xylonolactone to the corresponding acid. However, as shown by van Schie et al (1987), this spontaneous, non-enzymatic hydrolysis of xylononlactone is slow, which makes it difficult to attain an irreversible reaction. Another disadvantage of the method resides in the need for an added coenzyme, NADP.

In the U.S. Patent Specification No. 4,683,198, a maltose dehydrogenase isolated from *Staphylococcus* sp. is reported and its use for monosaccharide assays is suggested. However, no examples of, for instance, xylose determination are given. Moreover, the presented enzyme has a broad substrate spectrum and its activity towards oligomeric and even high molecular weight polymer is high. This makes the maltose dehydrogenase described in the prior art publication unsuitable for analytical purposes.

Xylose can also be quantified enzymatically by using xylose isomerase, which catalyzes the formation of xylulose from xylose. This method has, however, many disadvantages. The equilibrium of the reaction lies on the side of xylose and the formed xylulose is measured chemically by a complicated cystein-carbazole-method (Horecker 1974). Also a multistep method for xylose determination has been developed, in which both chemical and enzymatic steps have been combined (Williams and Withers 1986). Due to its complicity the proposed method is not practical enough for routine analyses. Deschatelets and Yu (1986) have developed a chemical method for xylose analysis but in that method toxic chemicals are used.

Finally, it should be mentioned that xylose can be electrochemically measured by using

systems based on pyranose oxidase (Olsson et al 1990) or mutarotase and an NAD+ dependent glucose dehydrogenase (Dominiguez et al 1988). In these methods, enzymatic reactions take place in a separate reactor and the compound detected at the electrode is either oxygen (Olsson et al 1990) or NADH (Dominiguez et al 1988). Oxygen detection is sensitive to naturally occurring levels of oxygen concentration. The method based on NADH detection, on the other hand, requires the addition of soluble NADH to the system. The NAD-linked glucose dehydrogenase has a very low turn-over number towards xylose. Thus, in order to analyze relative small concentrations of xylose, a high dose of enzyme is needed and even trace amounts of glucose contaminations in the sample disturb the analysis.

The present invention aims at eliminating the drawbacks of the prior art assay methods by providing a method for direct enzymatic determination of aldoses.

SUMMARY OF THE INVENTION

The invention is based on the idea of using a PQQ-linked aldose dehydrogenase enzyme for oxidizing the aldose to the corresponding aldonolactone. The enzyme is obtained from strains of gram-negative bacteria which are capable of oxidizing xylose or glucose and which have a specific membrane-bound aldose dehydrogenase. In particular, the enzyme comprises a xylose dehydrogenase isolated from the membranes of strains of the genus *Gluconobacter*.

The monomeric aldose units of oligomeric sugars can also be determined by the present method. The oligomers are first enzymatically hydrolyzed to the corresponding monomeric units. The glucose units present in the hydrolysate are then removed and separately determined, whereas the remaining hydrolysate is subjected to enzymatic oxidation with the aid of a PQQ-linked aldose dehydroganese in the presence of an electron acceptor.

The above-mentioned aldose dehydrogenase enzyme can be immobilized on the surface of a conductive electrode body and used as an aldose biosensor in a electrochemical method for the determination of aldoses.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The principle of the present method is depicted in Fig. 1. Thus, as will become apparent from the figure, the method is based on enzymatic oxidation of aldoses to the corresponding aldonolactones with the aid of a PQQ-linked aldose dehydrogenase. When the substrate, i.e. the aldose, is oxidized, it donates an electron to the cofactor of the aldose dehydrogenase (pyrroloquinoline quinone, PQQ), which further transfers the electron to an electron acceptor. The transfer of the electron from the cofactor PQQ to the electron acceptor is not an equilibrium reaction but an irreversible reaction. Thus, the amount of reduced electron acceptor will be proportional to the amount of aldose present in the sample.

Within the scope of this application, the term "aldose dehydrogenases" denotes enzymes capable of oxidizing aldoses by dehydrogenation. They can be active towards only one aldose. Usually they are, however, capable of oxidizing several aldoses and, possibly, also other mono-saccharides.

According to one preferred embodiment of the present invention, xylose is assayed by using a PQQ-linked aldose dehydrogenase. A particularly suitable aldose dehydrogenase can be obtained from the membranes of the *Gluconobacter oxydans* strain ATCC 621. This strain has been purified and characterized. The enzyme has a higher activity towards xylose than other isolated PQQ-linked aldose dehydrogenases earlier reported by Matsushita et al. (1980) and Dokter et al. (1986). The properties of the enzyme are summarized in Table 1.

Table 1. The properties of a PQQ-linked aldose dehydrogenase isolated from *Gluconobacter oxydans* strain ATCC 621

MW (kDa) (a)	Km (mM) xylose	Relative activity % (b)	Electron acceptor specificity % (c)
87	15.4	Xylose 100	DCIP-PMS 100
		Glucose 164	DCIP 38
		Galactose 105	NTB-PMS 261
		Mannose 85	Ferricyanide 24

a) determined by SDS-PAGE
b) DCIP-PMS as electron acceptor
c) xylose as substrate

The aldose dehydrogenase enzyme mentioned above is stable after purification even at room temperature for several days. The stability of the enzyme is illustrated by working example 1 given below. It can also easily be purified on large scale. The pH optimum of the enzyme clearly depends on the electron acceptor used (cf. below), for DCIP it is about 6 to about 6.5 and for DCIP-PMS it is about 8 (Example 1). It is not active towards xylo-oligomers (xylobiose, xylotriose, xylotetraose) (Example 6). In this respect it differes from the maltose dehydrogenase described in US Patent Specification No. 4,683,198, which is capable of oxidizing oligomeric substrates or even polymers, as well.

Due to the very simple xylose metabolism in *Gluconobacter* (Buchert and Viikari 1988), unpurified cell or membrane-extract or even whole cells or disrupted cells can also be used in the method of this invention.

The isolated aldose dehydrogenase from *G. oxydans* ATCC 621 is capable of oxidizing xylose, glucose, mannose, galactose and L-arabinose, as indicated by the data given in Table 1. Therefore, the interfering component(s), if any, must be removed from the sample before subjecting it to analysis for a certain aldose. It is known that glucose can be specifically removed by the combined action of glucose oxidase (EC 5.1.3.3), catalase (EC 1.11.1.6) and mutarotase (EC 5.1.3.3). Generally, samples which are to be assayed for their xylose content, e.g., hemicellulose hydrolyzates, mainly contain xylose and minor amounts of glucose. After the removal of glucose, the samples are first deproteinized by boiling them for a few minutes and then centrifuged. Thereafter, the xylose content of the samples can be specifically measured by the method described in this application. The glucose content of the sample can be assayed using the known glucose oxidase method prior to the removal (Bergmeyer 1974). If the sample contains xylo-oligomers, they can easily be hydrolyzed by the combined action of purified xylanase and β-xylosidase from *Trichoderma reesei* (Example 7) and thereafter quantified with the method of this invention.

The aldose dehydrogenase used in the present method, can also be isolated from other gram-negative bacteria, which are able of oxidizing xylose and which have a specific membrane-bound aldose dehydrogenase. The following examples of micro-organisms may be mentioned: *Acetobacter* sp., *Gluconobacter* sp., *Pseudomonas* sp., and *Acinetobacter* sp. (Dokter et al

1986, Buchert and Viikari 1988, Buchert 1990). We have earlier shown that in strains belonging to the genus *Gluconobacter* the xylose metabolism involves only a membrane-bound oxidation of xylose to xylonolactone (Buchert and Viikari 1988). Thus, all *Gluconobacter* strains capable of producing xylonic acid possess this membrane-bound aldose dehydrogenase (Buchert 1990). Examples of these kinds of strains are *G. oxydans* DMS 2003, ATCC 621-H, ATCC 19357 and ATCC 15163.

According to another preferred embodiment, the reduction of the electron acceptor is detected spectrophotometrically at an appropriate wavelength. In this embodiment, several different artificial electron acceptors can be used. Thus, the electron acceptor may be selected from the group consisting of dichlorophenol-indophenol (DCIP), DCIP combined with phenazinemethosulfate (PMS), ferricyanide, and nitrotetrazolium blue and PMS. It is preferred to use DCIP-PMS or NTB-PMS (Table 1). PMS functions as a electron mediator in the reaction. The wavelength for the spectrophotometrical determination of the reduction of the combination DCIP-PMS is preferably 600 nm, whereas in the case of NTB-PMS it is 540 nm. The pH of the activity reaction depends on the electron acceptor used. This feature will be described in more detail below in Example 1.

The present assay method is linear when the measured xylose concentration in the assay system lies in the range from 0 to 2.5 mM (sample concentrations 0 - 5 g/l, cf. Example 2). For glucose the linear area corresponds to concentrations in the range from 0 to 0.2 mM (sample concentrations 0 - 0.5 g/l, cf. Example 4). When the glucose concentration of the assay system is from 0 to 0.2 mM, xylose, arabinose, galactose or mannose do not disturb the assay if the molar ratio of glucose to these aldoses is 1 or more.

According to a third preferred embodiment of the invention, the reduction of the electron acceptor is electrochemically measured. This feature is illustrated in working examples 8, 9 and 10 described below. The electron acceptor will function as an electron transfer mediator between the PQQ and the electrode. Species which can function as electron acceptors and which can be electrochemically oxidized, preferably derivatives of ferrocene, can be used as mediators. The measurement potential depends on the electron acceptor used.

In the course of the work leading up to the present invention it surprisingly turned out that the

PQQ-linked aldose dehydrogenases described herein have properties which make them especially suitable for aldose biosensor applications. When NAD(P) dependent dehydrogenases are utilized, a soluble coenzyme must be added to the solution or the coenzyme must be immobilized in the system, which may impair the biochemical activity of the coenzyme. The PQQ-dehydrogenases, however, have their coenzyme tightly bound to the enzyme through van der Waals-forces or covalent bonds. Thus, according to a fourth preferred embodiment based on the above described electrochemical measurement of the reduction of the electron acceptor, the aldose dehydrogenase is immobilized onto or in close proximity of the electrode used in the electrochemical detection. The immobilization can be carried out by, e.g., covalent bonding, adsorption or entrapment onto the surface of the conductive body of the electrode. The immobilized enzyme electrode works as an aldose biosensor. The electron acceptor can be immobilized on the electrode by same methods as the enzyme.

It is known in the prior art to use flavin-linked enzymes for carbohydrate assays. The present invention provides considerable benefits in comparison to that prior art. Thus, a clear advantage of PQQ-dehydrogenases over flavoproteins is their oxygen insensitivity. Oxygen has a strong influence on the enzyme activity of flavoproteins and experiments have to be performed at constant oxygen concentration. If artificial electron acceptors are used with flavoproteins any oxygen present can affect the measurement. These problems can be avoided when PQQ-dehydrogenases are used. Moreover, electrochemical rate constants for the transfer of electrons between the PQQ-dehydrogenases and ferrocene-based mediators are generally considerably higher than those for other oxidoreductases. For xylose determination this method is very well suited for samples having concentrations usually present in biotechnical research (i.e. the xylose concentration to be measured lies in the range from about 1 to about 5 g/l).

The other benefits of the present method are the simplicity of the assay and the short incubation times. The reduction of the electron acceptors can be monitored either as end-point detection or according to the initial reaction rate. The end-point method is especially suitable for samples containing very small amount of aldoses. Also, the reaction mixture does not need any added cofactors and the measurements can be performed in visible light. The enzyme has high activity for xylose compared to other membrane-bound dehydrogenases. Due to the different Km values for xylose and glucose, the measurement range for xylose is from 0 to 5

g/l and for glucose from 0 to 0.5 g/l. Still other benefits of this method are the non-pathogenity of the producing strain and the relatively high activity towards xylose as compared to for example to *Acinetobacter calcoaceticus* glucose dehydrogenase (Dokter et al 1986).

The present method is especially suitable for the analysis of monomeric aldoses, but it can be combined to analysis of oligomeric sugars. The oligomeric samples can be enzymatically pretreated and thereafter assayed as described herein. Samples containing xylo-oligosaccharides can be analyzed by the enzymatic method after hydrolysis of the oligomers to monosaccharides by the combined action of purified xylanase and β-xylosidase. This embodiment is illustrated by working example 6. Samples containing cello-oligosaccharides can be first hydrolyzed to monomers by β-glucosidase and purified cellulases (endoglucanase and cellobiohydrolase) and thereafter the glucose concentration can be measured by the method according to the present invention. The measurement principle can easily be applied also to an electrochemical detection method.

BRIEF DESCRIPTION OF THE DRAWINGS

In the accompanying drawings:

FIG. 1 shows the principle of the assay according to the invention;

FIG. 2 shows the stability of the aldose dehydrogenase enzyme extracted from the membranes of the *Gluconobacter oxydans* strain ATCC 621;

FIG. 3 shows the pH range of stability of the aldose dehydrogenase;

FIG. 4 shows the pH optimum of the aldose dehydrogenase for different electron acceptors;

FIG. 5 shows the results of an assay of D-xylose in samples containing 0 to 5 g xylose per liter;

FIG. 6 shows standard curves for the detection of galactose, mannose, xylose and L-arabinose;

FIG. 7 shows the detection range for glucose;

FIG. 8A shows a HPLC chromatogram for a quantitative assay of xylo-oligosaccharides; and FIG. 8B shows a corresponding chromatogram after hydrolysis with xylanase and β-xylosidase;

FIG. 9 shows the detection range for xylose and glucose in an electrochemical assay using soluble ferrocene carboxylic acid as a mediator; and

FIG. 10 shows the detection range for xylose in an electrochemical xylose determination using a dimethylferrocene modified electrode.

The following examples describe the invention in more detail.

Example 1 - Stability and pH optimum of the ADH.

Stability of ADH was found to increase during the purification of the enzyme. The membrane extract purified with one chromatographic step retained over 50 % of its activity during 2 week storage at room temperature (Fig. 2). The enzyme was most stable at pH range 3.5 - 6 (Fig. 3). pH optimum of the ADH was found to be 6.5 in the electrochemical xylose measurement with dimethylferrocene as an electron acceptor and 8 for soluble enzyme DCIP-PMS as an electron acceptor (Fig. 4). However pH 6.0 or 6.5 was preferably used in the measurements because of the better stability of the enzyme.

Example 2 - Xylose assay

D-xylose in samples containing 0 - 5 grams xylose per liter was measured at 600 nm by detecting the increase of the absorbance as a result of the reduction of DCIP-PMS.

The assay system consists of:

880 µl of potassium phosphate buffer pH 6 (50 mM)

20 µl DCIP (2.5 g/l)

20 µl PMS (0.828 g/l)

80 µl aldose dehydrogenase (specific activity 85 µkat/g)

80 µl of xylose containing sample (xylose concentration 0 - 5 g/l)

All components except the enzyme are added to a cuvette, thereafter the enzyme was added, mixed, and the reaction is followed at 600 nm. The assay results are presented in Fig. 5.

Example 3 - Standard curves for various monosaccharides

Standard curves for the detection of galactose, mannose, xylose and L-arabinose are depicted in Fig. 6. Assay conditions were as described in example 2.

Example 4 - Glucose assay

Glucose was measured as described in example 2. The sample contained 0 - 0.5 g/l glucose. Linear working range for the detection of glucose was thus 0 - 0.5 g/l (Fig. 7).

Example 5 - Determination of xylose in mixtures containing xylose and glucose

Xylose was determined in a mixture containing 1 g/l xylose and 0,5 g/l glucose. The glucose was first measured by the GOD method and then specifically removed from the sample by the action of catalase and glucose oxidase and mutarotase.

To 1 ml of the sample 1000 U of glucose oxidase (Sigma) and 1000 U of catalase (Sigma) and 418 U of mutarotase (Sigma) were added and the mixture was incubated at 30 °C for 20 min by bubbling the sample with air for about 10 minutes. After incubation the sample was deprotonized by immersing it into a boiling water bath for 2 minutes, and centrifuged. The xylose in the sample was measured as described in Example 2 with the aid of a standard curve.

The results are given in the following table:

Table 2. Xylose assay

Compound	Initial concentration g/l	After glucose removal g/l	
		HPLC	this method
Xylose	1.0	1.02	1.02
Glucose	0.5	0	-

Example 6 - The activity of the aldose dehydrogenase towards oligosaccharides

The activity of the aldose dehydrogenase towards xylobiose, xylotriose and xylopentaose was also measured. The assay system consisted of 960 µl of potassium phosphate buffer pH 6.5, 40 µl of sample (concentration in the sample 6 g/l), 40 µl of PMS, 20 µl of DCIP, 20 µl of the enzyme with an activity of 13 µkat/ml). Aldose dehydrogenase did not oxidize any of the oligomers tested, although xylose in similar concentrations was oxidized.

The results are given in table 3:

Table 3 - The assays:

Compound	Initial substrate conc. [g/l]	Measured concentration [g/l]
Xylose	6	6
Xylobiose	6	0
Xylotriose	6	0
Xylotetraose	6	0

Example 7 - Quantitative measurement of xylo-oligosaccharides

A sample containing xylo-oligosaccharides (HPLC-chromatogram in Fig. 8A) had a total carbohydrate concentration (as measured by the sulfuric acid-orcinol method) of 1.31 mg/ml.

1 ml of this sample was hydrolyzed with purified *Trichoderma reesei* xylanase, 13 nkat (Tenkanen et al, submitted) and β-xylosidase 2 nkat, (corresponding to a total enzyme dose of 10 000 nkat of xylanase and 1 000 nkat of β-xylosidase per g of xylo-oligosaccharides), at 45 °C for 24 h. After hydrolysis the xylose concentration of the sample was analyzed by HPLC (Fig. 8B) and by the method described in example 2. The amount of total oligosaccharides was (after hydrolysis) as analyzed by HPLC 1.25 g/l and by the present method 1.25 g/l. Thus, the method according to the invention can be used for quantitative determination of oligosaccharides.

Example 8 - Electrochemical xylose measurement

The aldose dehydrogenase was immobilized on graphite electrodes treated with heat by covalent bonding. The electrodes were first immersed in 20 $mgml^{-1}$ solution of 1-ethyl-3-(3-dimethylamino-propyl)carbodi-imide in milliQ water on ice for 80 minutes. The electrodes were then rinsed with a 10 mM sodium acetate buffer pH 5.5 and immersed in an aldose dehydrogenase solution (specific activity 130 μkat/g) for 2 h on ice. Subsequently the electrodes were washed in 10 mM acetate buffer pH 5.5.

This enzyme electrode was used as the working electrode in an electrochemical cell together with a platinum wire as counter electrode and an Ag/AgCl electrode as reference electrode. The electrodes were poised at fixed potential in stirred solution of a 50 mM phosphate buffer (pH 6) containing soluble ferrocene carboxylic acid. Xylose was injected as a concentrated solution to the stirred electrolyte solution in small aliquots and the resulting change in current was measured. After the addition of xylose the current increased, which indicates that the electrode is working as a xylose sensor. The linear working range of the electrode was found to extend to a final xylose concentration of 5 g/l in the electrolyte when soluble ferrocene carboxylic acid was used as a mediator (Fig. 9).

Example 9 - Electrochemical measurement of glucose

Glucose was measured electrochemically with a system described in example 8. The linear working range for glucose extended in this experiment to about 0.5 g/l (Fig. 9).

Example 10 - Electrochemical xylose measurement with dimethylferrocene modified electrode

The electrode was modified with dimethylferrocene as described in example 8. Subsequently the aldose dehydrogenase was immobilized as described in example 8. The electrode was poised at fixed potential in a stirred 50 mM phosphate buffer (pH 6.5). Measurement of xylose was performed as described in example 7. With a dimethylferrocene modified electrode it was possible to expand the working range of the xylose sensor to 15 g/l (Fig. 12).

It will now be apparent to those skilled in the art that other embodiments, improvements, details and uses can be made consistent with the letter and spirit of the foregoing disclosure and within the scope of this patent, which is limited only by the following claims, construed in accordance with the patent law, including the doctrine of equivalents.

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Claims:

1. A method for the determination of aldoses, according to which method the aldose which is to be determined is enzymatically oxidized to the corresponding aldonolactone with the aid of an aldose dehydrogenase in the presence of an electron acceptor, the reduction of the electron acceptor being determined as a measure of the amount of aldose, c h a r a c t e r i z e d by using a pyrroloquinoline quinone (PQQ) -linked aldose dehydrogenase as enzyme.

2. The method as claimed in claim 1, c h a r a c t e r i z e d by using a PQQ-linked aldose dehydrogenase produced by a gram-negative bacterial strain, which is capable of oxidizing xyloses and which has a specific membrane-bound aldose-dehydrogenase.

3. The method as claimed in claim 2, c h a r a c t e r i z e d by using a PQQ-linked aldose dehydrogenase produced by a bacterial strain selected from the group consisting of strains belonging to the genera *Gluconobacter*, *Acetobacter*, *Pseudomonas* and *Acinetobacter*.

4. The method as claimed in claim 3, c h a r a c t e r i z e d by using a PQQ-linked aldose dehydrogenase produced by the strain *Gluconobacter oxydans* ATCC 621.

5. The method as claimed in any of claims 2 to 4, c h a r a c t e r i z e d by using an isolated and purified enzyme.

6. The method as claimed in any of claims 2 to 4, c h a r a c t e r i z e d by using unpurified cells, membrane-extract, whole cells or disrupted cells of the aldose dehydrogenase producing bacterial strain.

7. The method as claimed in any of the previous claims, which comprises determining spectrophotometrically the reduction of the electron acceptor, c h a r a c t e r i z e d by using an electron acceptor selected from the group consisting of dichlorophenol-indophenol (DCIP), DCIP combined with phenazinemethosulfate (PMS), ferric cyanide, and nitrotetrazolium blue combined with PMS.

8. The method as claimed in claim 7, which comprises using dichlorophenol-indophenol combined with phenazinemethosulfate as electron acceptor, c h a r a c t e r i z e d by determining the reduction of the electron acceptor at a wavelength of 600 nm.

9. The method as claimed in claim 7, which comprises using nitrotetrazolium blue combined with phenazinemethosulfate as electron acceptor, c h a r a c t e r i z e d by determining the reduction of the electron acceptor at a wavelength of 540 nm.

10. The method as claimed in any of claims 1 to 6, which comprises determining the reduction of the electron acceptor electrochemically, c h a r a c t e r i z e d by using a derivative of ferrocene as electron acceptor.

11. The method as claimed in claim 10, c h a r a c t e r i z e d by using a ferrocene derivative selected from the group consisting of ferrocene carboxylic acid and dimethyl-ferrocene.

12. The method as claimed in any of the previous claims for the determination of xylose in a sample containing a mixture of aldoses, c h a r a c t e r i z e d by removing any glucose present in the sample before the xylose assay.

13. The method as claimed in claim 12, c h a r a c t e r i z e d by subjecting the sample to the action of at least one of the enzymes selected from the group consisting of glucose oxidase, catalase and mutarotase.

14. The method as claimed in claims 12 or 13, c h a r a c t e r i z e d by determining enzymatically the amount of glucose removed by methods known per se.

15. The method as claimed in any of the previous claims for determining the monomeric aldose units in oligomeric sugars, c h a r a c t e r i z e d by the steps of

- enzymatically hydrolyzing the oligomeric sugars to the corresponding monosaccharides with the aid of at least one enzyme selected from the group consisting of xylanase, β-xylosidase, cellulase and β-glucosidase to form a hydrolysate.
- subjecting, if desired, the hydrolysate to the combined action of glucose oxidase, catalase

and mutarotase in order to remove any glucose present in the hydrolysate,

- determining the amount of glucose, if any, removed from the hydrolysate by methods known per se,

- subjecting the hydrolysate to an enzymatic oxidation with the aid of an PQQ-linked aldose dehydrogenase in the presence of an artificial electron acceptor, and

- determining the reduced electron acceptor as a measure for the monomeric xylose units present in the oligomeric sugars.

16. An electrode for use in an electrochemical method for the determination of aldoses, c h a r a c t e r i z e d in that it comprises a conductive body essentially covered with a layer containing an PQQ-linked aldose dehydrogenase.

17. The electrode as claimed in claim 16, c h a r a c t e r i z e d in that the layer also contains an electron acceptor.

18. The electrode as claimed in claim 17, c h a r a c t e r i z e d in that the layer comprises an PQQ-linked aldose dehydrogenase and dimethylferrocene immobilized on the surface of the electrode body.

19. The electrode as claimed in claim 18, c h a r a c t e r i z e d in that the aldose dehydrogenase is immobilized by covalent bonding, adsorption or entrapment to the surface of the electrode body.

AMENDED CLAIMS

[received by the International Bureau on 30 March 1992 (30.03.92); new claim 1 added; claims 2-4 replaced by new claims 3-5; claims 1 and 4 replaced by new claim 6; claims 5-14 replaced by new claims 7-16; claim 15 replaced by new claim 2; claims 16-19 replaced by new claims 17-20 (3 pages)]

1. A membrane-bound aldose-dehydrogenase, having the cofactor PQQ and further having the following characteristics:

a) a molecular weight of 87,000 Da as determined by SDS-PAGE, and

b) specific activity towards xylose, glucose, mannose and galactose.

2. A method for determining the monomeric aldose units of oligomeric sugars, c h a r a c t e r i z e d by the steps of

- enzymatically hydrolyzing the oligomeric sugars to the corresponding monosaccharides with the aid of at least one enzyme selected from the group consisting of xylanase, β-xylosidase, cellulase and β-glucosidase to form a hydrolysate,
- subjecting, if desired, the hydrolysate to the combined action of glucose oxidase, catalase, and mutarotase in order to remove any glucose present in the hydrolysate,
- determining the amount of glucose, if any, removed from the hydrolysate by methods known per se,
- subjecting the hydrolysate to an enzymatic oxidation with the aid of an PQQ-linked aldose dehydrogenase in the presence of an artificial electron acceptor, and
- determining the reduced electron acceptor as a measure for the monomeric xylose units present in the oligomeric sugars.

3. The method as claimed in claim 2, c h a r a c t e r i z e d by using a PQQ-linked aldose dehydrogenase produced by a gram-negative bacterial strain, which is capable of oxidizing xyloses and which has a specific membrane-bound aldose-dehydrogenase.

4. The method as claimed in claim 3, c h a r a c t e r i z e d by using a PQQ-linked aldose dehydrogenase produced by a bacterial strain selected from the group consisting of strains belonging to the genera *Gluconobacter*, *Acetobacter*, *Pseudomonas* and *Acinetobacter*.

5. The method as claimed in claim 4, c h a r a c t e r i z e d by using a PQQ-linked aldose

dehydrogenase produced by the strain *Gluconobacter oxydans* ATCC 621

6. A method for the determination of aldoses, according to which method the aldose which is to be determined is enzymatically oxidised to the corresponding aldonolactone with the aid of an aldose dehydrogenase in the presence of an electron acceptor, the reduction of the electron acceptor being determined as a measure of the amount of aldose, c h a r a c t e r i z e d by using a pyrroloquinoline quinone (PQQ) -linked aldose dehydrogenase produced by the strain *Gluconobacter oxydans* ATCC 621.

7. The method as claimed in claim 6, c h a r a c t e r i z e d by using an isolated and purified enzyme.

8. The method as claimed in claim 6, c h a r a c t e r i z e d by using unpurified cells, membrane-extract, whole cells or disrupted cells of the aldose dehydrogenase producing bacterial strain.

9. The method as claimed in any of claims 6 - 8, which comprises determining the reduction of the electron acceptor spectrophotometrically, c h a r a c t e r i z e d by using an electron acceptor selected from the group consisting of dichlorophenol-indophenol (DCIP), DCIP combined with phenazinemethosulfate (PMS), ferric cyanide, and nitrotetrazolium blue combined with PMS.

10. The method as claimed in claim 9, which comprises using dichlorophenol-indophenol combined with phenazinemethosulfate as electron acceptor, c h a r a c t e r i z e d by determining the reduction of the electron acceptor at a wavelength of 600 nm.

11. The method as claimed in claim 9, which comprises using nitrotetrazolium blue combined with phenazinemethosulfate as electron acceptor, c h a r a c t e r i z e d by determining the reduction of the electron acceptor at a wavelength of 540 nm.

12. The method as claimed in any of claims 6 to 8, which comprises determining the reduction of the electron acceptor electrochemically, c h a r a c t e r i z e d by using a

derivative of ferrocene as electron acceptor.

13. The method as claimed in claim 12, c h a r a c t e r i z e d by using a ferrocene derivative selected from the group consisting of ferrocene carboxylic acid and dimethyl-ferrocene.

14. The method as claimed in any of claims 6 to 13 for the determination of xylose in a sample containing a mixture of aldoses, c h a r a c t e r i z e d by removing any glucose present in the sample before the xylose assay.

15. The method as claimed in claim 14, c h a r a c t e r i z e d by subjecting the sample to the action of at least one of the enzymes selected from the group consisting of glucose oxidase, catalase and mutarotase.

16. The method as claimed in claims 14 or 15, c h a r a c t e r i z e d by determining enzymatically the amount of glucose removed by methods known per se.

17. An electrode for use in an electrochemical method for the determination of aldoses, c h a r a c t e r i z e d in that it comprises a conductive body essentially covered with a layer containing an PQQ-linked aldose dehydrogenase.

18. The electrode as claimed in claim 17, c h a r a c t e r i z e d in that the layer also contains an electron acceptor.

19. The electrode as claimed in claim 18, c h a r a c t e r i z e d in that the layer comprises an PQQ-linked aldose dehydrogenase and dimethylferrocene immobilized on the surface of the electrode body.

20. The electrode as claimed in claim 19, c h a r a c t e r i z e d in that the aldose dehydrogenase is immobilized by covalent bonding, adsorption or entrapment to the surface of the electrode body.

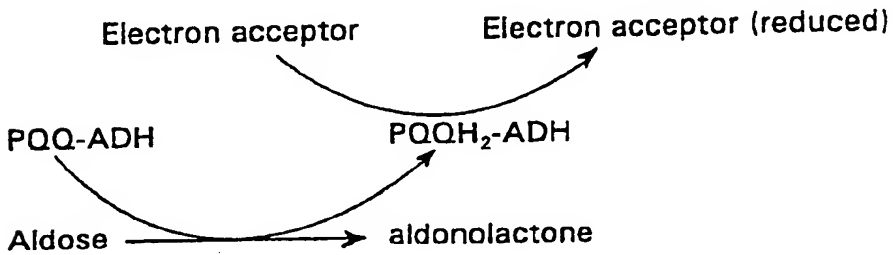

FIG. 1. The principle of the aldose determination

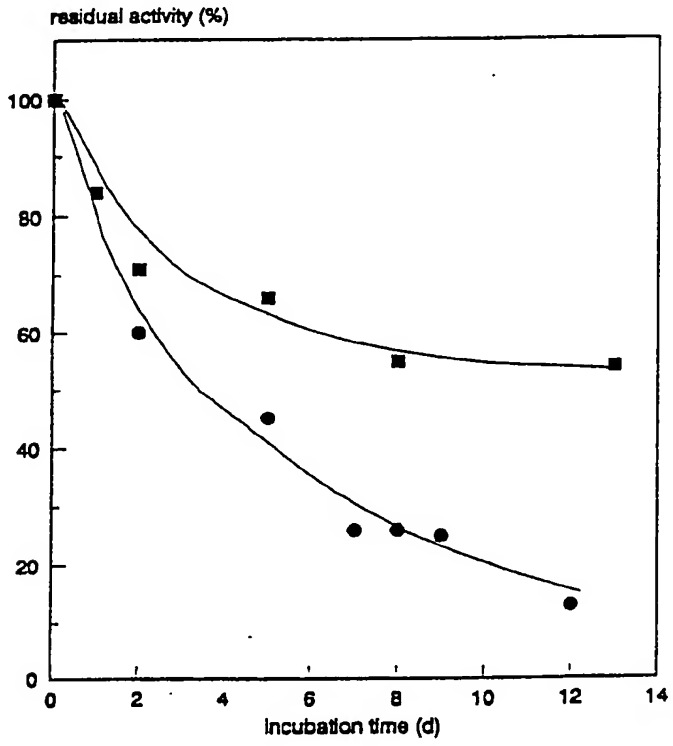

FIG. 2 Stability of the aldose dehydrogenase at room temperature at different purification steps

- ● membrane extract
- ■ after 1 chromatographic purification step

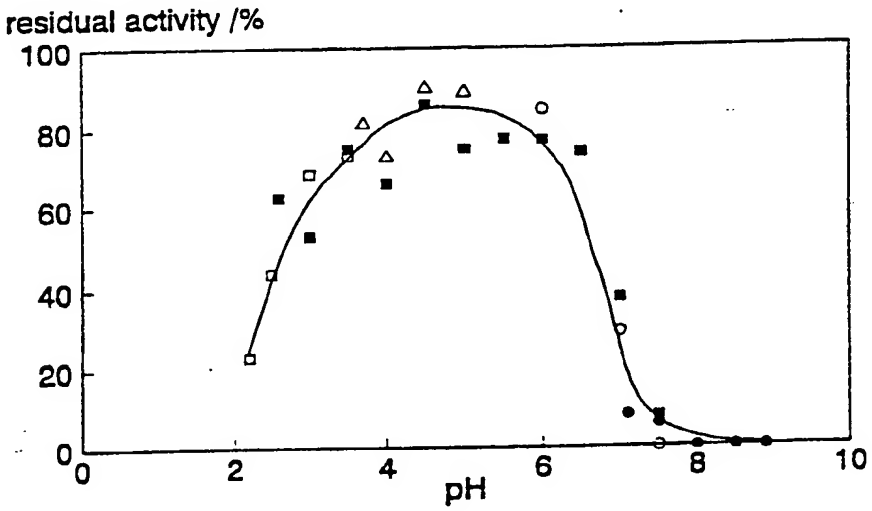

FIG. 3 pH stability of the aldose dehydrogenase

● Tris-HCl
○ potassium phosphate - NaOH
□ glysine-HCl
△ sodium acetate
■ McIlvaine

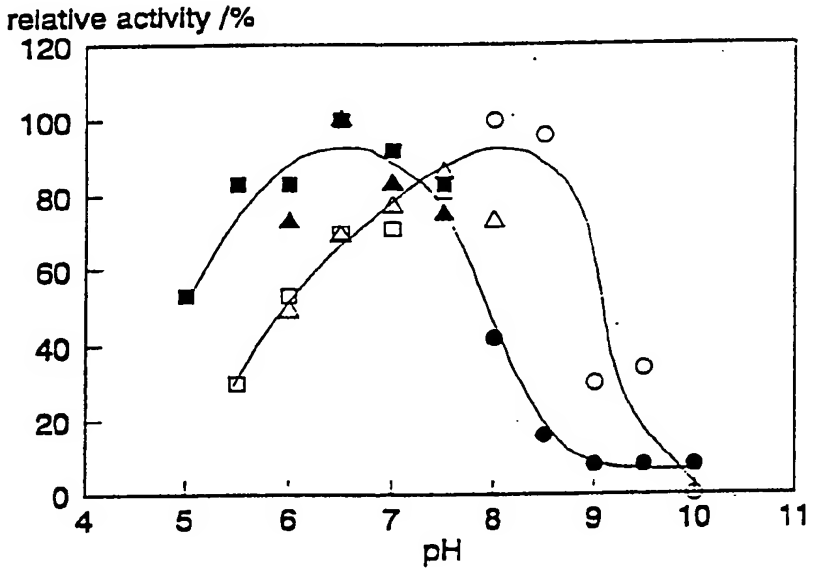

FIG. 4 pH optimum of the aldose dehydrogenase
■▲● dimethylferrocene as an electron acceptor
□△○ DCIP-PMS as an electron acceptor
■□ McIlvaine
▲△ potassium phosphate
● ○ Clark and Lubs

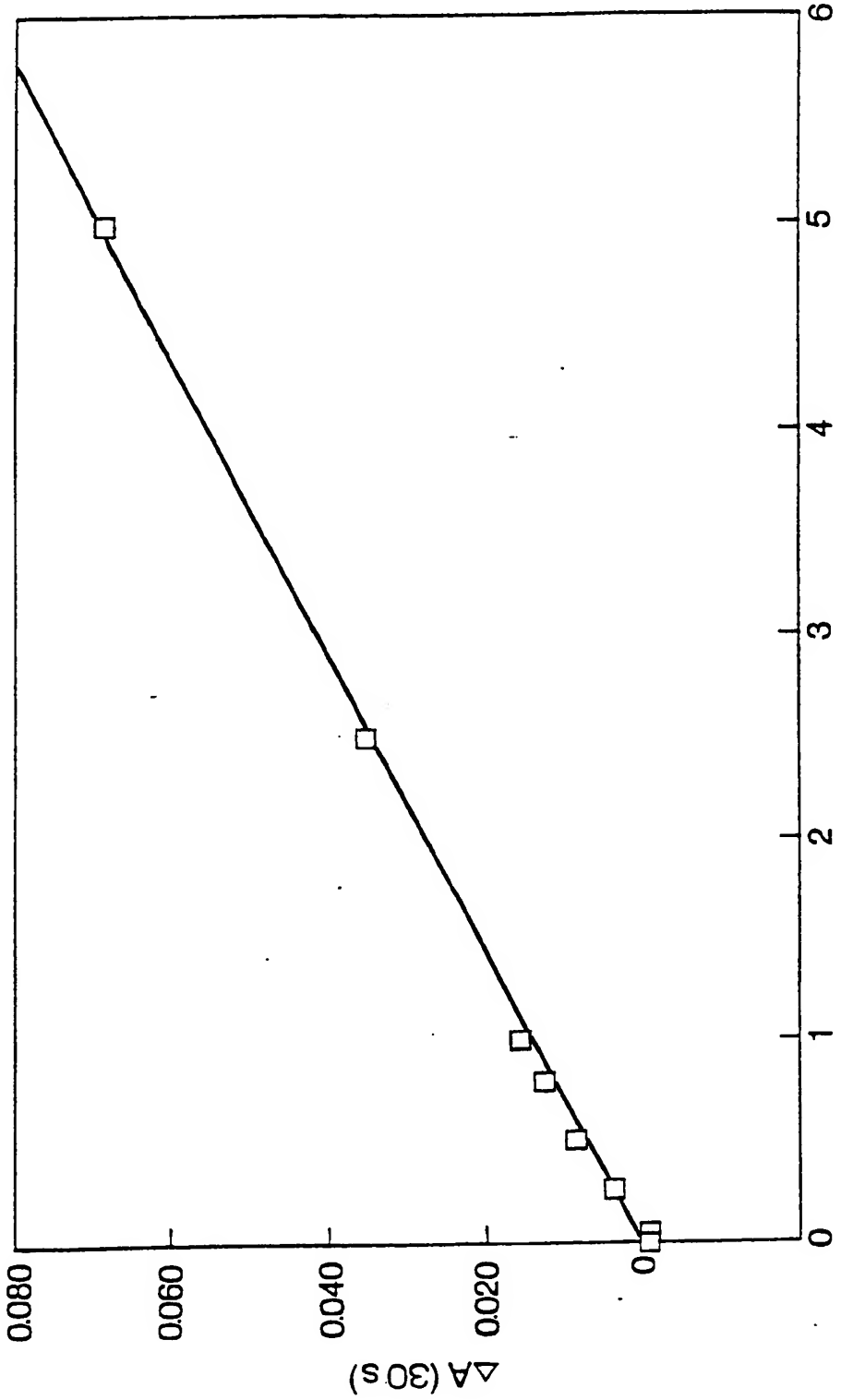
ΔA (30 s)
0.080
0.060
0.040
0.020
0
0
1
2
3
4
5
6
Xylose concentration in sample g/l)

FIG. 5

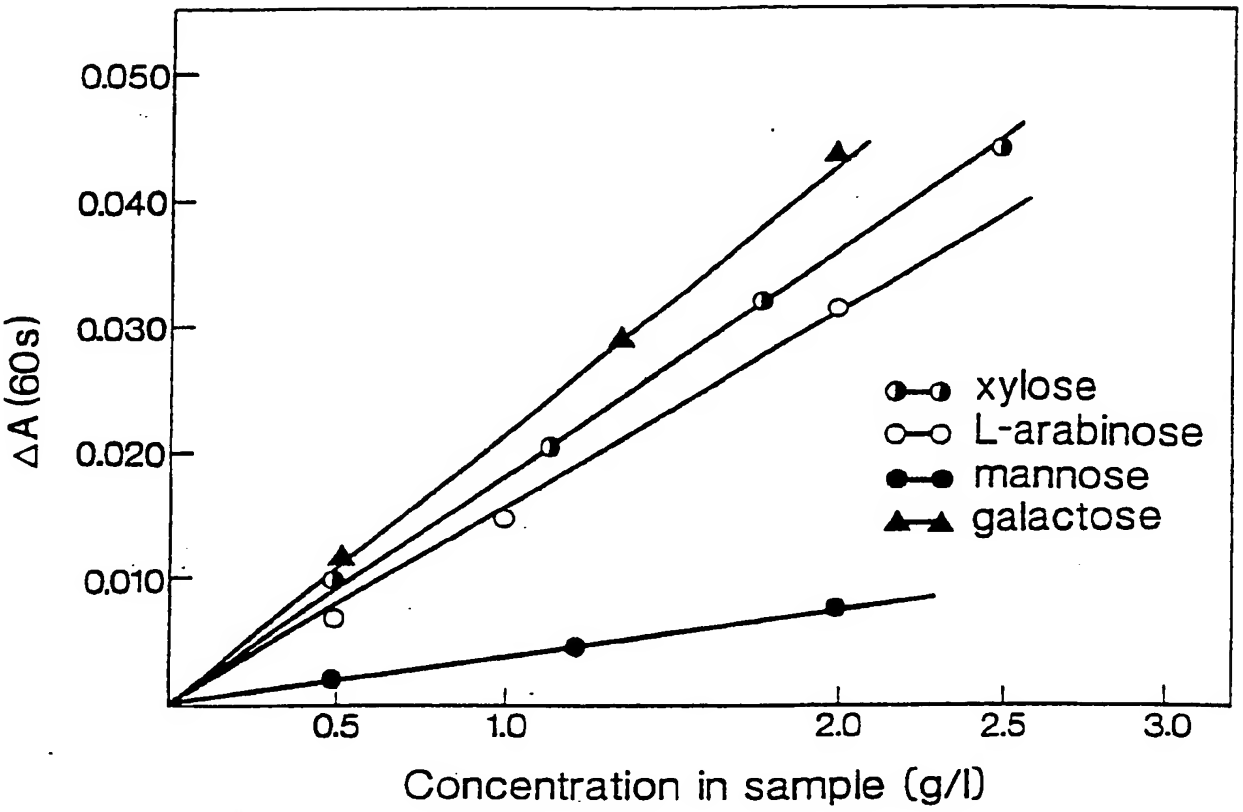
0.050
0.040
0.030
0.020
0.010
ΔA (60s)
0.5
1.0
2.0
2.5
3.0
Concentration in sample (g/l)
xylose
L-arabinose
mannose
galactose

FIG. 6

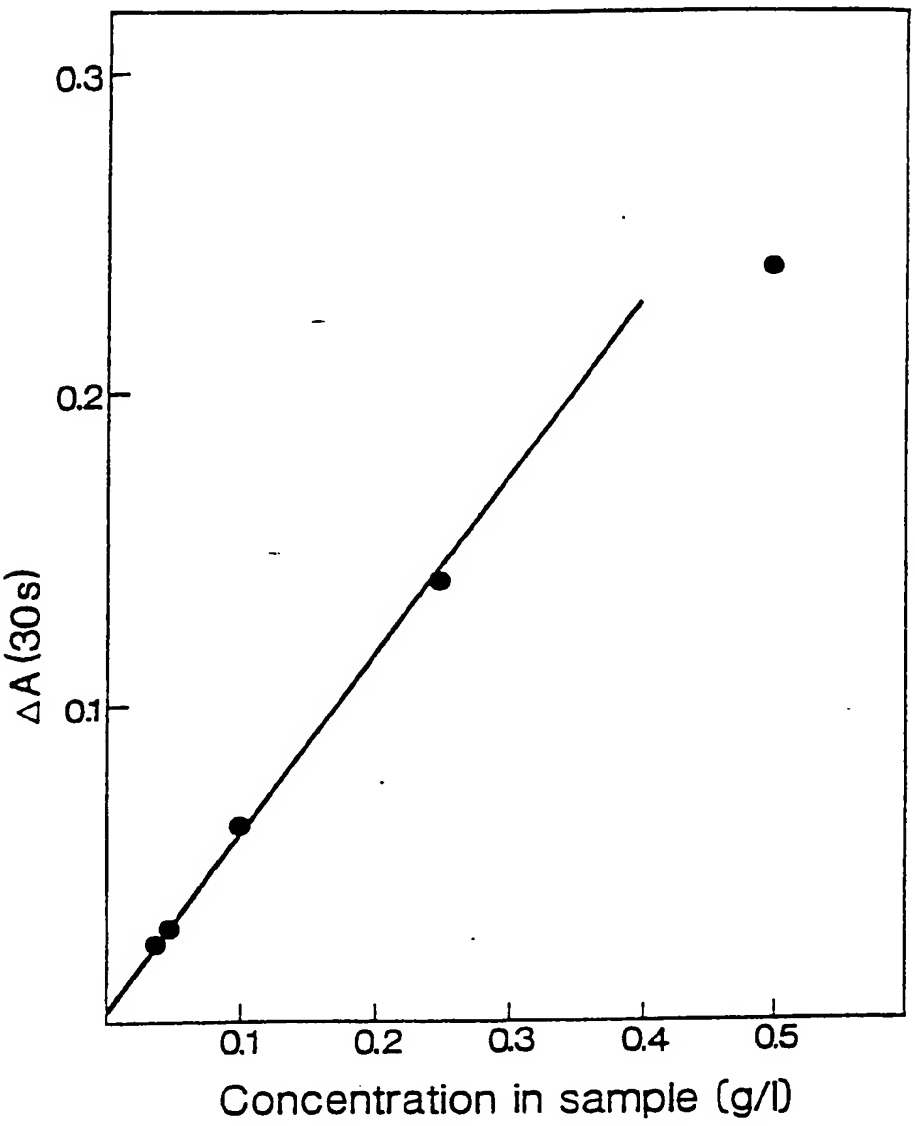

FIG. 7

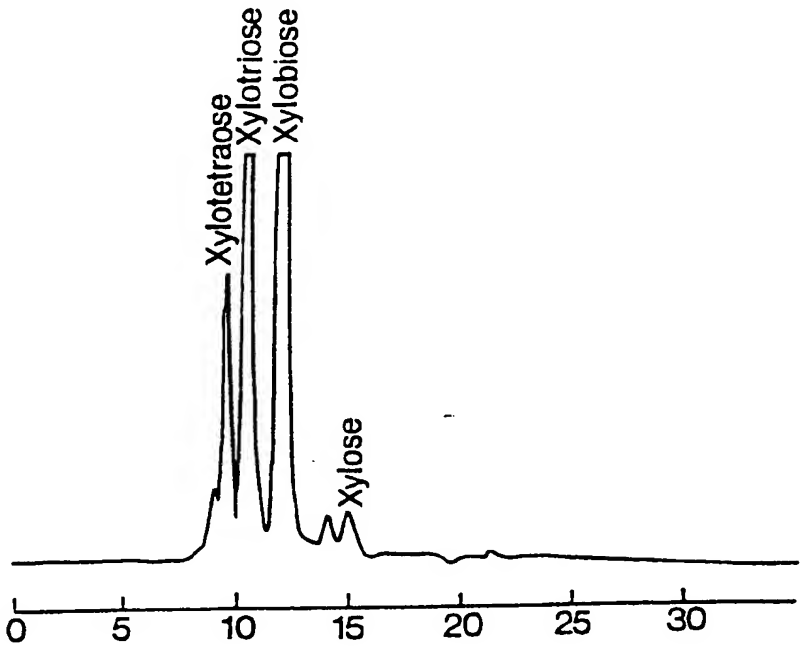
Xylotetraose
Xylotriose
Xylobiose
Xylose
0
5
10
15
20
25
30

FIG. 8A

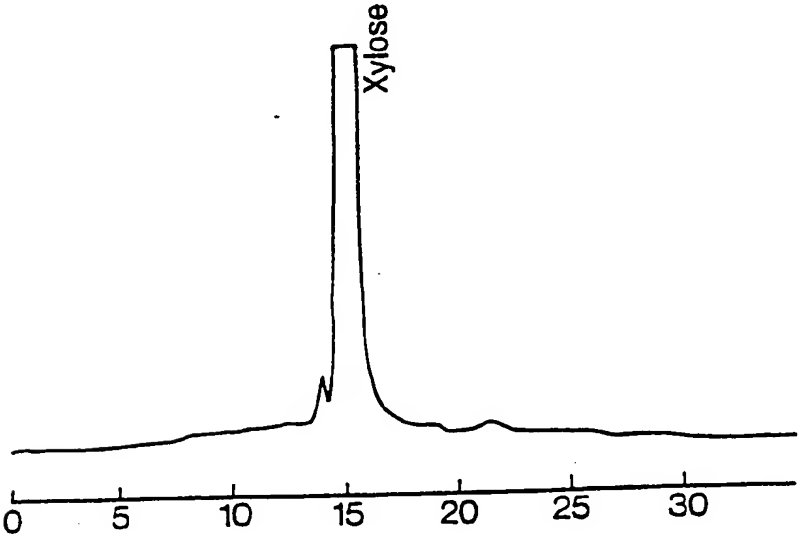
Xylose
0
5
10
15
20
25
30

FIG. 8B

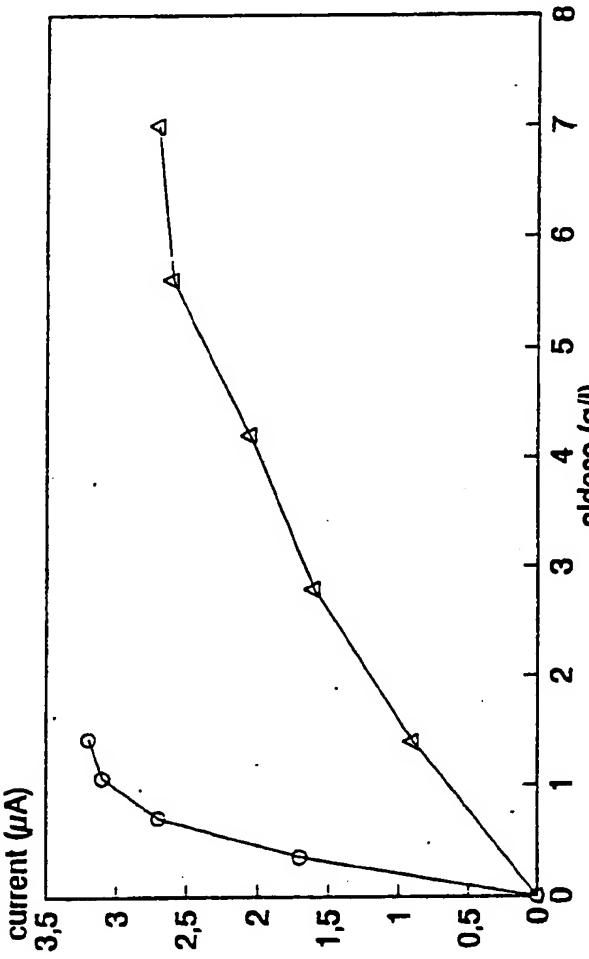

FIG. 9 Electrochemical measurement of xylose and glucose with aldose dehydrogenase modified electrode

glucose xylose

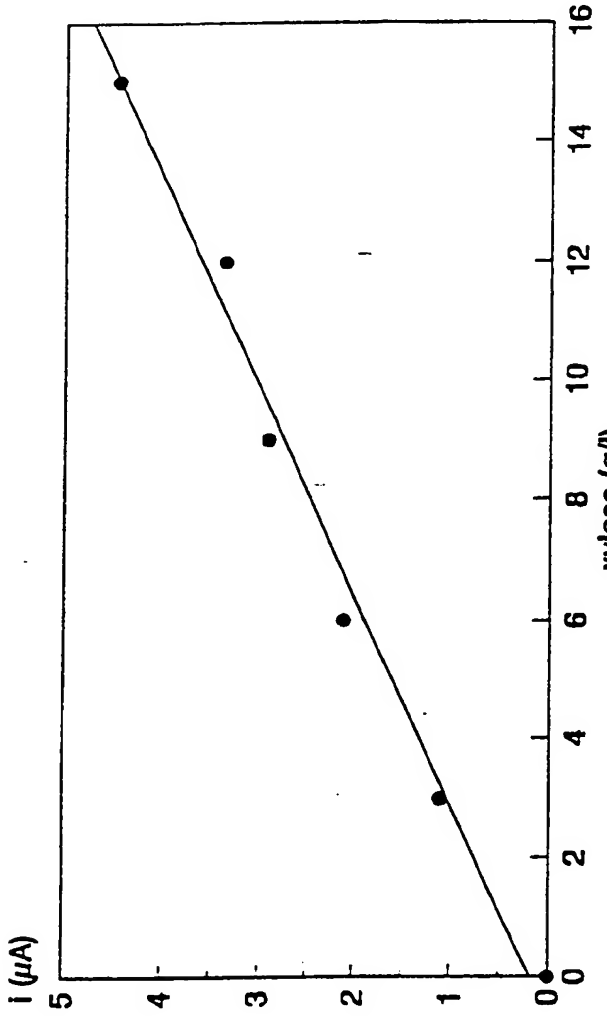

FIG. 10 Electrochemical measurement of xylose with dimethylferrocene and aldose dehydrogenase modified electrode

INTERNATIONAL SEARCH REPORT

International Application No PCT/FI 91/00324

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 Q 1/32, C 12 M 1/40//C 12 N 9/04

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 12 N; C 12 Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	EP, A1, 0094161 (IMPERIAL CHEMICAL INDUSTRIES PLC) 16 November 1983, see the whole document	1-19
Y		1-19
Y	EP, A2, 0332120 (NAKANO VINEGR CO., LTD.) 13 September 1989, see page 2, line 3 - line 14; page 4, line 6 - line 18; page 4, line 28 claims	1-4,6
X	EP, A2, 0125137 (GENETICS INTERNATIONAL, INC.) 14 November 1984, see page 5, line 15 - line 21; page 6, line 2 - line 10 claims 1-6,9-10,16,18-19 and 21	1-5,10-11,16-19

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
25th February 1992	1992 -03- 02
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Carl Olof Gustafsson

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
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Y	Agric. Biol. Chem., Vol. 44, No. 7, 1980 Kazunobu Matsushita et al.: "Membrane-bound D-Glucose Dehydrogenase from Pseudomonas sp.:Solubilization, Purification and Characterization", see page 1505 - page 1512	4,12
A	EP, A1, 0078636 (GENETICS INTERNATIONAL, INC.) 11 May 1983, see examples 9-13	1-19
A	Chemical Abstracts, volume 105, no. 19, 10 November 1986, (Columbus, Ohio, US), Dokter, Paulet al.: "Purification and characterization of quinoprotein glucose dehydrogenase from Acinetobacter calcoaceticus L.M.D. 79.41. ", see page 330, abstract 167677z, & Biochem. J. 1986, 239(1), 163- 167	1-19
A	Chemical Abstracts, volume 110, no. 10, 6 March 1989, (Columbus, Ohio, US), Dominguez, E. et al.: "A flow-injection system for the amperometric determination of xylose and xylulose with coimmobilized enzymes and a modified electrode ", see page 865, abstract 87729f, & Anal. Chim. Acta 1988, 213(1), 139-150	1-19
A	Dialog Information Services, File 357, Dialog accession no. 072782, Turner A P F et al: "Enzymatic analysis using quinoprotein dehydrogenases - characterization of carbodiimide-immobilized glucose-dehydrogenase (on graphite foil) enzyme electrode for glucose assay, (conference paper)", & Ann. N.Y. Acad. Sci. (501, 283-87) 1987	1-19

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/FI 91/00324

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the Swedish Patent Office EDP file on 30/12/91
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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		US-A-	4925212	90-05-15
EP-A2- 0125137	84-11-14	AU-B-	564494	87-08-13
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		AU-D-	2775284	85-01-31
		AU-D-	2775384	84-11-08
		AU-D-	2775484	84-11-08
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		EP-A-	0125867	84-11-21
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		JP-A-	60017347	85-01-29
		JP-A-	60017360	85-01-29
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		US-A-	4758323	88-07-19
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		EP-A-	0127958	84-12-12
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		EP-A-	0351892	90-01-24
FI-B- 84625	91-09-13	NONE		
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		AU-D-	8972282	83-05-12
		CA-A-	1212146	86-09-30
		DE-A-	3278334	88-05-19
		JP-B-	2059424	90-12-12
		JP-A-	58099746	83-06-14
		US-A-	4545382	85-10-08